PROTOCOL: Automated Protein Digestion

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03
- Dithiothreitol (DTT), Sigma-Aldrich, Cat. No. 43819-5G
- Iodoacetamide (IAM), Sigma-Aldrich, Cat. No. I1149-25G
- 50 mM Tris solution pH 8.0, diluted from Invitrogen, Cat. No. 15568-025
- Trypsin 0.5 µg/µl in 50 mM acetic acid, Promega, Cat. No. V5113
- Trifluoroacetic acid (TFA), Sigma-Aldrich, Cat. No. T6508-25ML
- Agilent 96LT-180uL Filter Tips, Agilent Technologies, Cat. No. 19477-042
- 96-Well 2.2mL Deep Well plate, VWR, Cat. No. 82006-448
- 96-Well skirted PCR plate, Bio-rad, Cat. No. MSP9601
- 384-Well Deep Well Microplate, Greiner Bio-One, Cat. No. 781270
- 12-Column High Profile Reagent Reservoir, Axygen, Cat. No. RES-MW12-HP
- Axygen -80°C Rated Foil Seal, Axygen, Cat. No. PCRAS200
- pH Test Strips, JT Baker, Cat. No. 4393-01
- Breath-EASIER seal, Diversified Biotech, Cat. No. BERM-2000

Assets

- BioMicroLab Rack Thawing Station
- Agilent LT-BRAVO Automated Liquid Handling Platform
- Eppendorf microcentrifuge
- Eppendorf plate shaker

Procedure

Conditions to digest one 96-well plate of samples.

Six milliliters each of 100 mM DTT and 200 mM iodoacetamide were prepared fresh on day of use. Two milliliters of trypsin was thawed and stored on wet ice. Twenty milliliters of 25% TFA were prepared in a 50 mL Falcon tube. Bravo was turned on prior to loading the device file “Bravo with Heated Shakers.dev” and the protocol “LysateDigestion (8M Urea).pro”. The heater/shaker was set to 37C.

Reagents were manually pipetted into a 384 well plate using a multichannel pipet according to the plate map in Figure 1.

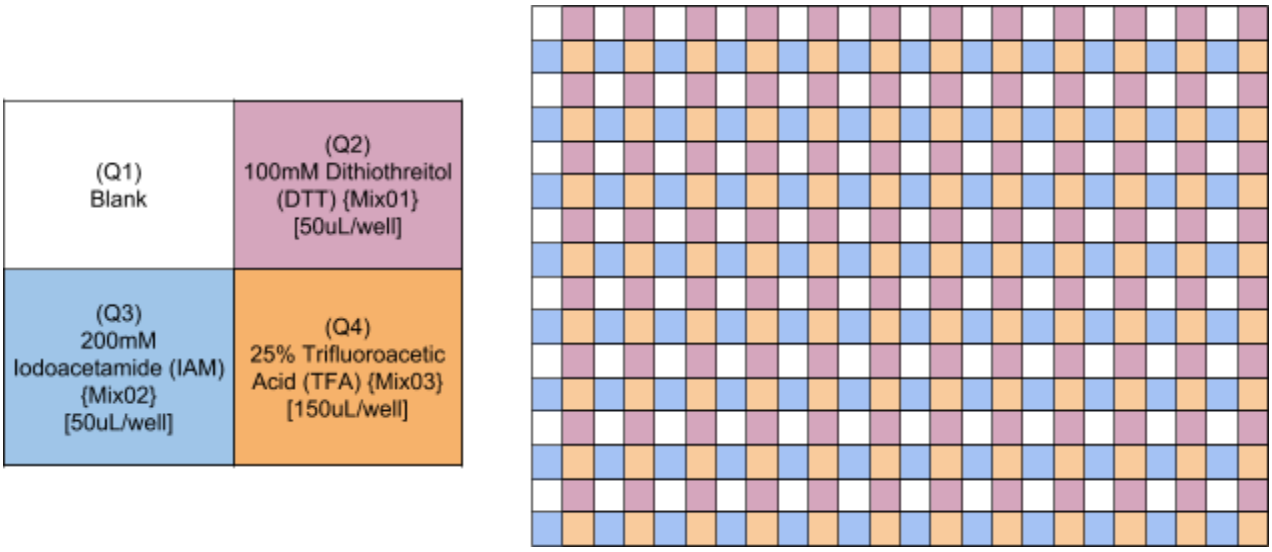

Figure 1. Plate map and configuration of reduction, alkylation and quenching reagents.

Trypsin was added (20µL) to each well of the 96-well PCR plate. Both plates, 384 well reagent plate and 96 well trypsin plate, were covered with foil and kept at 4C on wet ice until use. The 12-column high profile reagent reservoir was filled with 150 mL of 50 mM Tris pH 8.0. The 96 well plate containing cell lysate samples, the reagent, trypsin and Tris buffer plates were placed onto the Bravo deck along with the racks of pipet tips and waste collection plates as shown in Figure 2.

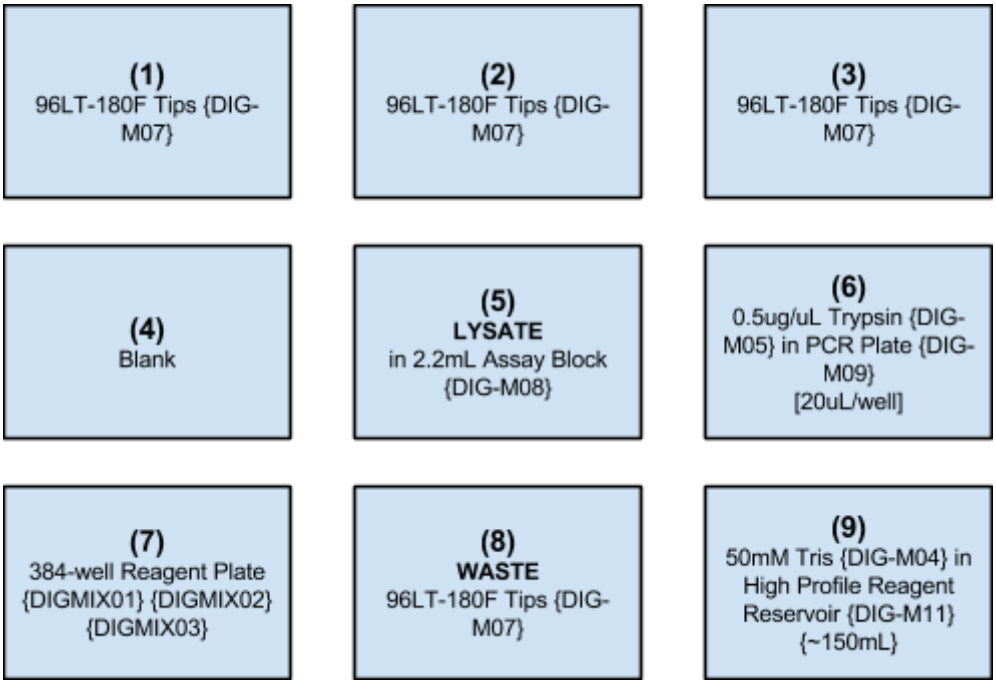

Figure 2. Bravo deck layout and configuration of plates and pipet tips.

The method and device files were loaded on the Vworks software version 4 controlling the LT-BRAVO. The method was first ‘simulated’ to validate the protocol by toggling to ‘simulation is on’. During ‘simulation’ the entire protocol is performed in silico to check for programming errors. Since this does not actually move the head to verify that the proper plates or pipet tips were installed, the layout of the plates on the Bravo deck was manually verified before starting the digestion protocol. To initiate the simulation, ‘Start’ was pressed which activates the ‘Run Configuration Wizard’. After pressing ‘Finish’ the number of ‘Columns’ was set to 12, the number in the 96 well sample plate, was entered into the

dialog box titled, ‘Set Initial Values for Variables’. After entering the column number ‘OK’ was selected to perform the simulation. After any detected ‘errors’ were fixed the simulation was toggled ‘off’ and the Bravo was covered with the light impermeable fabric which allows the robotic arm to operate while preventing exposure to ambient light. ‘Go’ was pressed to start the protocol, 12 was entered into the column number dialog box to start the automated digestion.

After approximately 1 h, all the digestion reagents, 25 uL DTT, 25 uL IAA, 10 uL trypsin were added by the Bravo, the programmed stop sequence (refer to full Bravo-LT Digestion Program in Appendix 1) paused the Bravo-LT. The lysate plate was removed from the deck and sealed with aluminum foil seal to reduce evaporation during overnight digestion, returned to the heater/shaker position 4 on the Bravo deck and incubated and mixed for 15 h at 37C and 800 RPM. The next day, the lysate plate was removed from the Bravo, vortexed and centrifuged for 1 min at 1,400 RPM and returned to the same location (#4) on the Bravo after removing the foil seal. ‘Go’ was pressed and the Bravo performed the next operation, the reaction in each well was quenched by adding 75 uL of 25% TFA. The lysate plate was removed from Bravo, vortexed and centrifuged for 1 min at 1,400 RPM. The foil seal was removed and pH of less than 3.0 was verified by pH test strips. Lysate plate was kept at 4C or on wet ice until the next step, desalting.

Appendix 1. Bravo-LT Digestion Protocol

1. Define Variables
    1.1. Columns = 12
    1.2. ReagentVol = 25
2. Reduction
    2.1. Set head mode to all barrels
        2.1.1. task.Headmode=”1,2,8,”+Columns;
        2.1.2. This script can be used in “Advanced Settings” in conjunction with “Define Variables” to set the number of “Columns” to the appropriate number.
    2.2. Tips on from Position 1.
    2.3. Aspirate 25uL from quadrant 2 of position 7. (DTT)
        2.3.1. Volume = ReagentVol
        2.3.2. Post-aspirate volume = 5
        2.3.3. Distance from well bottom = 0.5
        2.3.4. Well selection = 1 selection: quadrant 2
    2.4. Dispense contents of tips to position 5. (LYSATE)
        2.4.1. Distance from well bottom = 2
    2.5. Tips off in Position 1.
    2.6. Shake plate from Postion 5 at Position 4.
        2.6.1. 37degC, 800rpm, 30 minutes
3. Place plate at Position 5.
4. Alkylation
    4.1. Set head mode to all barrels
        4.1.1. task.Headmode=”1,2,8,”+Columns;
        4.1.2. This script can be used in “Advanced Settings” in conjunction with “Define Variables” to set the number of “Columns” to the appropriate number.
    4.2. Tips on from Position 1.
    4.3. Aspirate 25uL from quadrant 3 of position 7. (IAM)
        4.3.1. Volume = ReagentVol
        4.3.2. Post-aspirate volume = 5
        4.3.3. Distance from well bottom = 0.5
        4.3.4. Well selection = 1 selection: quadrant 3
    4.4. Dispense contents of tips to position 5. (LYSATE)
        4.4.1. Distance from well bottom = 2
    4.5. Mix 180uL at Position 5.
        4.5.1. Mix cycles = 10
        4.5.2. Distance from well bottom = 2
    4.6. Tips off in Position 1.
5. Incubate at Position 5 for 30 minutes in the dark.
6. Dilution
    6.1. Set head mode to all barrels
        6.1.1. task.Headmode=”1,2,8,”+Columns;
        6.1.2. This script can be used in “Advanced Settings” in conjunction with “Define Variables” to set the number of “Columns” to the appropriate number.
    6.2. Tips on from Position 2.
    6.3. Loop 7 times changing tips every 7 times.
    6.4. Aspirate 165uL from Position 9. (50mM Tris)
        6.4.1. Post-aspirate volume = 10

6.4.2. Distance from well bottom = 1
6.5. Dispense contents of tips to Position 5. (LYSATE)
6.5.1. Distance from well bottom = 2.
6.6. Loop End.
6.7. Tips off at Position 2.

7. Trypsin Digest
7.1. Set head mode to all barrels
7.1.1. task.Headmode="1,2,8,"+Columns;
7.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
7.2. Tips on from Position 3.
7.3. Aspirate 10uL from Position 6. (Trypsin)
7.3.1. Distance from well bottom = 1
7.4. Dispense contents of tips to Position 5. (LYSATE)
7.4.1. Distance from well bottom = 2
7.5. Tips off at Position 3.
7.6. Wait for user to press the "Go" button.
7.6.1. "Cover digest plate in foil, then press GO."
7.7. Shake plate from Position 5 at Position 4.
7.7.1. 37degC, 800rpm, 15 hours.

8. Stop Digest
8.1. Set head mode to all barrels
8.1.1. task.Headmode="1,2,8,"+Columns;
8.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
8.2. Wait for user to press the "Go" button.
8.2.1. "Spin down plate, remove foil cover, then press GO."
8.3. Tips on from Position 8.
8.4. Aspirate 75uL from quadrant 4 of Position 7. (25% TFA)
8.4.1. Post-aspirate volume = 5
8.4.2. Distance from well bottom = 0.5
8.4.3. Well selection = 1 selection: quadrant 4
8.5. Dispense contents of tips to Position 5. (LYSATE)
8.5.1. Distance from well bottom = 2
8.6. Mix 180uL at Positon 5.
8.6.1. Mix cycles = 5
8.6.2. Distance from well bottom = 1
8.7. Tips off at Position 8.